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First 3D reconstruction of the rhizocephalan root system using MicroCT



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ABSTRACT

Parasitic barnacles (Cirripedia: Rhizocephala) are highly specialized parasites of crustaceans. Instead of an alimentary tract for feeding they utilize a system of roots, which infiltrates the body of their hosts to absorb nutrients. Using X-ray micro computer tomography (MicroCT) and computer-aided 3D-reconstruction, we document the spatial organization of this root system, the interna, inside the intact host and also demonstrate its use for morphological examinations of the parasites reproductive part, the externa. This is the first 3D visualization of the unique root system of the Rhizocephala in situ, showing how it is related to the inner organs of the host. We investigated the interna from different parasitic barnacles of the family Peltogastridae, which are parasitic on anomuran crustaceans. Rhizocephalan parasites of pagurid hermit crabs and lithodid crabs were analysed in this study.

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1. Introduction

Parasitic barnacles (Rhizocephala) are parasites of crustaceans, mainly decapods. The adult female parasite consists of a sac-shaped reproductive body (the externa), which is connected to a system of roots situated inside the host's body (the interna). The interna is the sole trophic organ of the parasite and in some species forms an almost mycelia-like structure that spreads throughout the body of the host (Bresciani and Høeg, 2001; Høeg and Lützen, 1995). The externa, which in most species is situated on the pleon of the host (Fig. 1), is clad with a flexible cuticle. It consists of a mantle, a mantle cavity that harbours the egg mass, and the visceral mass containing the ovary. The visceral mass usually also contains a pair of receptacles, each hosting an extremely small dwarf male, which acts as a functional testicle (Høeg, 1995; Walker, 2001).

The interna of the Rhizocephala is a unique organ in Arthropoda. Comparable trophic structures are only found in the thoracican barnacles *Anelasma*, parasitic on sharks, and *Rhizolepas*, parasitic on polychaetes (Day, 1939; Rees et al., 2014), as well as in some parasitic copepods (Boxshall and Harrison, 1988). The gross morphology of the interna varies significantly between higher taxonomic categories in the Rhizocephala, with some species having a system of randomly branching rootlets, while others exhibit a more organized root system. Also the extension of the interna varies greatly, with some species infiltrating the whole body while others are locally confined (Bresciani and Høeg, 2001).

While the first rhizocephalan was already discovered in 1836 (Thompson, 1836), it was only observed more than 20 years later that the parasites not only consist of the sac-shaped externa, but also of an extensive part inside its host, the interna (Anderson, 1858). Anderson noticed “an innumerable quantity of copper-coloured tubules, which ramify through the whole body” when studying the rhizocephalan *Peltogaster paguri* on the hermit crab *Pagurus bernhardus* (Fig. 1c). He also correctly recognized that “the function of these tubes is evidently to supply the body of the parasite with nourishment”. Müller (1862) incorporated the discovery of the internal root system in the name of the taxon and erected the Rhizocephala, derived from the Greek words roots (rhiza) and head (cephale), referring to the interna and externa respectively. He also provided the first drawings of fragments of the root system. A monograph on the rhizocephalan *Sacculina carcini* by Delage (1884), including descriptions of the interna, must have inspired Haeckel, who included an illustration of this parasite into his famous lithographic book “Kunstformen der Natur” (Haeckel, 1904), where the parasite is displayed with an extensive root system inside its host, the green crab *Carcinus maenas*. Haeckel must have made the illustration solely based on descriptions and not on direct observation, which is also indicated by a slightly wrong attachment site of the externa. And while Haeckel's drawing is thus rather an abstract illustration, modified versions have been widely used to illustrate the interna of the Rhizocephala (e.g. Brusca and Brusca, 2003; Høeg and Lützen, 1985). Only few other illustrations are showing the overall organization of the root system in their hosts (Lützen, 1981; Pérez, 1937), and the organization of the interna has yet never been visualized three-dimensionally along with the associated organs of the host.

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Fig. 1. Photographs of living anomuran hosts with attached rhizocephalan externae. (a) The small lithodid crab *Hapalogaster mertensii*, dorsal view. (b) *H. mertensii*, ventral view, with an externa of *Briarosaccus tenellus* situated on the soft ventral side of the pleon. (c) The hermit crab *Pagurus bernhardus* with an externa of *Peltogaster paguri* situated on the pleon, which is usually protected by an empty gastropod shell. (d) The hermit crab *Pagurus hirsutisculus* with an externa of *Peltogaster* sp. situated on the pleon. Abbreviations: ex, externa.

Due to the delicacy and irregular morphology of the interna, it is problematic to accurately reconstruct its extremely complex branching structure inside the host. The study of the spatial organization of the rhizocephalan interna has to date been based on dissections, scanning electron microscopy (SEM), and on histological serial sections of embedded specimens, from which the topology of the interna has been inferred (Bortolini and Alvarez, 2008; Bresciani and Høeg, 2001; Isaeva et al., 2012; Lützen, 2002; Lützen, 1981; Shukalyuk, 2002; Shukalyuk et al., 2001).

Classical dissection will by opening the integument of the crab obscure the intimate association between interna and the organs of the host, making it extremely difficult to reconstruct the natural three-dimensional shape of the interna. In most species of the rhizocephalan family Peltogastridae the interna is coloured bright green due to the pigment billiverdin (Fox, 1953), which makes it easily distinguishable from the host's tissue when dissected fresh. But the internae of most other species are whitish, making them in addition difficult to detect and distinguish from the host's tissue in dissections.

SEM has been employed to document the spatial morphology of rhizocephalan internae, but only visualized fractions, and not in situ along with the associated organs inside the host (Bresciani and Høeg, 2001; Bresciani and Lützen, 1980).

While resin or paraffin embedding, on the other hand, provides an option to keep the internal organs in their natural position within the host, 3D reconstructions from serial sections would be an almost impossible task due to the irregular structure of the interna and the size of the hosts.

Recently studies have precisely mapped the organization and extent of the delicate cardiovascular system in situ of a number of arthropods (Huckstorf and Wirkner, 2011; Klußmann-Fricke et al., 2012; Wirkner and Prendini, 2007; Wirkner and Richter, 2004) and in particular anomuran crustaceans (Keiler et al., 2013; Keiler et al., 2015a; Keiler et al., 2015b), using X-ray micro computer tomography (MicroCT), which encouraged us to test this method on rhizocephalans parasitizing anomuran hosts. In the present study we employ MicroCT in combination with computer-aided 3D-reconstruction for the first time to visualize the spatial organization of the rhizocephalan interna inside an intact

host. In addition to the visualization of the parasite's interna, we also demonstrate the efficacy of MicroCT on the sac shaped reproductive body, the externa. The examination of the externa usually requires the destruction of the specimen. MicroCT, on the other hand, provides a non-disruptive method for the investigation of the externa.

2. Materials and methods

2.1. Taxon sampling

For this study five different rhizocephalan species of the family Peltogastridae, which are exclusively parasitic on anomuran crustaceans, were investigated for the organization of the parasite's root system. Species scanned with the MicroCT were *Peltogaster curvatus* on the hermit crab *Pagurus prideaux* (1 specimen) from western Norway; *Peltogaster boschmai* on the hermit crab *Discorsopagurus schmitti* (2 specimens) from Washington State, USA; *Peltogaster* sp. on the hermit crab *Pagurus pubescens* (4 specimens) from the Svalbard Archipelago, Norway; *Peltogaster* sp. on the hermit crab *Pagurus hirsutisculus* (2 specimens) (Fig. 1d) from Southeastern Alaska, USA; and *Briarosaccus tenellus* on the small lithodid crab *Hapalogaster mertensii* (3 specimens) (Fig. 1a, b) from Southeastern Alaska, USA.

2.2. Specimen preparation

Specimens were narcotized by adding clove oil to the seawater and fixed in Bouin's fixative or 4% formalin buffered in seawater. The fixed specimens were rinsed and stored in 70% ethanol prior further treatment. The specimens were contrast stained, and dried by freeze-drying or critical point drying prior scanning. For critical point drying, the specimens were dehydrated in an ascending ethanol series, transferred to a 1% iodine solution in 100% ethanol for 24 h for contrast staining, and critical point dried in CO₂ using an Emitech K850 (UK). For freeze drying, the specimens were hydrated in a descending ethanol series, and contrast stained for 24 h using IKI stain (Metscher, 2009). After being washed in water, the specimens were frozen at −20 °C and subsequently freeze-dried using a UniCryo MC2L (UniEquip, Germany).

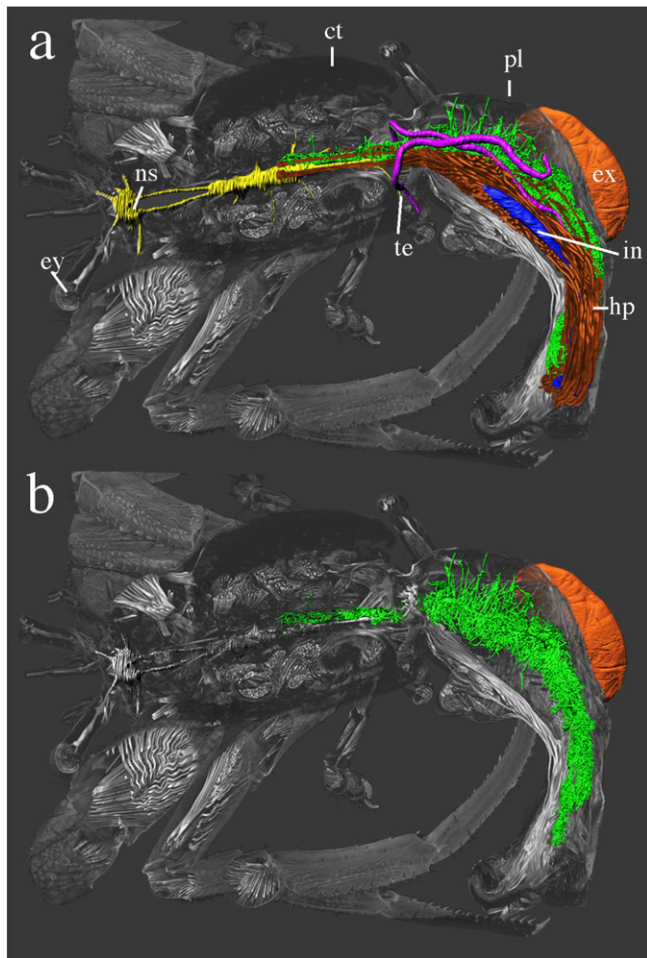


Fig. 2. The interna of *Peltogaster* sp. in the hermit crab *Pagurus pubescens* reconstructed using MicroCT, dorsal view of the host. (a) Main trunk of the interna (highlighted in green) in situ inside the host, shown together with the host's organs. Fine rootlets in the carapace region are not shown. Externa marked in orange. The hermit crab is virtually cut open from the dorsal side, and the digestive tract in the cephalothorax is removed to show the underlying interna and nervous system. Hepatopancreas highlighted in brown, intestine in blue, nervous system in yellow, and testes in purple. (b) View as in (a), with the highlighted organs of the pleon virtually removed. Abbreviations: ct, cephalothorax; ey, eye; ex, externa; hp, hepatopancreas; in, intestine; ns, nervous system; pl, pleon; te, testes.

2.3. MicroCT

X-ray imaging of the dried specimens was performed at the University of Rostock with a Phoenix nanotom1 (Phoenix x-ray, GE Sensing & Inspection Technologies) high-resolution MicroCT system in high-resolution mode, using the programme phoenix datos x acquisition (target: molybdenum, mode: 0–1; performance: ca. 8–13 W; number of projections: 720–1440; detector-timing: 1000–3000 ms; voxel size ca. 10–30 μm). A volume file was generated using the software phoenix datos x reconstruction and a stack of virtual sections exported with the software VGStudio Max (Volume Graphics, Heidelberg, Germany).

2.4. 3D reconstruction

3D-reconstruction was carried out using image stacks of virtual sections with the software Imaris 7.6 (Bitplane). A scene was created in the programme module “surpass”, and the volume - rendering function chosen to visualize the entire data set. The contours of organs of the host studied were marked with polygons on the virtual cross-section,

using the “surfaces” function. The resulting 3D reconstructions are therefore termed “surface renderings”. Different functions (“isoline” and “distance”) were used for segmentation. Stacks of polygons were visualized by surface renderings. The surface models of some organs were used to expose the volume region corresponding to the organ in question (“mask channel”) and depicted as “blend projection.” In a further optional step, an automatic surface model was calculated (“automatic creation”) to obtain a more realistic surface model of the organ. To reconstruct the interna, small body regions of the host containing parts of the interna were marked and treated in the same way as the host's organs, and the interna surface was calculated using the automatic surface model (“automatic creation”). Merging the partial surfaces assembled the complete interna surface. Colours were assigned to the individual organs. The clipping tool was used to cut the volume in different angles, exposing the interna and internal organs inside the specimens. The final figure plates were assembled using Adobe Photoshop CS5.

3. Results and discussion

3.1. Reconstruction of the rhizocephalan interna using MicroCT

The interna is a very delicate structure, with an extremely thin cuticle (Bresciani and Høeg, 2001), thus the applicability of MicroCT on this organ was unknown prior to our study. Our results show that MicroCT is a powerful method for visualizing the rhizocephalan root systems in situ inside of their crustacean hosts. The visibility of the root system, and especially the differentiation from the surrounding host tissue, varied between the individual scanned specimens and also between different parts of the host's body. Differentiation problems might be due to general fixation issues, variable levels of contrast differences between the interna and surrounding host tissue, or might occur in regions in which the interna is packed tightly to the host's organs.

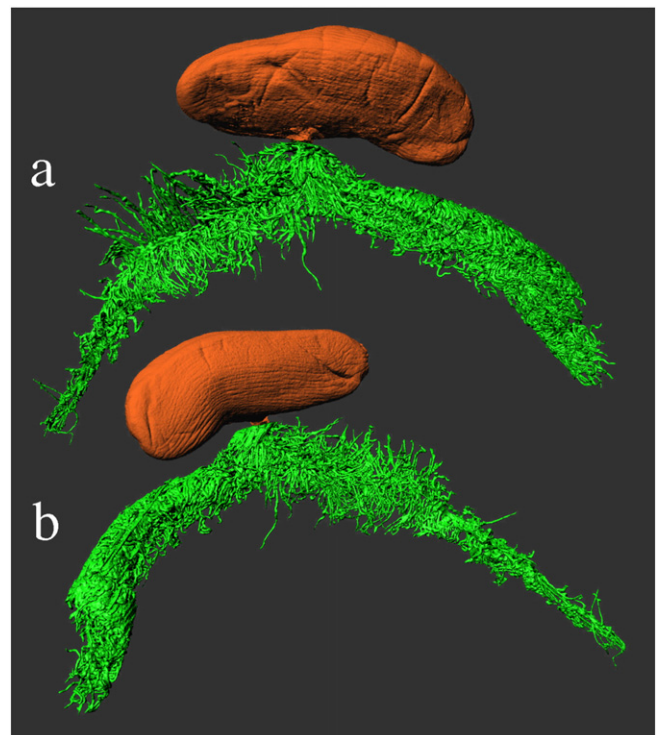


Fig. 3. Reconstruction of the rhizocephalan *Peltogaster* sp. from the hermit crab *Pagurus pubescens* using MicroCT, virtually removed from its host (see Fig. 2). Externa highlighted in orange, main trunk of interna highlighted in green. (a) Right side view of the externa; (b) left side view of the externa.

While the delicate cardiovascular system of arthropods can be studied by injecting resin, greatly enhancing the contrast when investigated using MicroCT (Keiler et al., 2013), we used solely iodine to enhance the contrast levels between different tissues, since injected resin would unlikely distribute throughout the root system. Using iodine as contrasting medium, the whole specimen and not specifically the interna is treated. This might explain the different levels of possible discrimination between interna and host organs.

3.2. The interna of *Peltogaster*

The gross morphology of the interna of *Peltogaster* spp. in the present study is in agreement with the earlier descriptions of *P. paguri*

(Bresciani and Høeg, 2001; Pérez, 1937; Pérez, 1931). The interna can be divided into a main trunk, situated in the pleon of the hermit crab, and a network of irregularly branching rootlets, which are spreading between the organs of the cephalothorax. At the attachment site of the externa, the main trunk of the interna divides into two main branches (Figs. 2, 3). One branch is directed posteriorly in the host, following its soft pleon. The other, anteriorly directed branch extends into the posterior cephalothorax region of the host, where it splits up into a few branches, which again are branching into finer rootlets between the host's organs. While the interna is clearly visible in the pleon of most scanned hermit crab specimens, it is less visible and harder to discriminate from the host's organs in the cephalothorax region. The main trunk, previously described as resembling a horse's tail (Bresciani and Høeg,

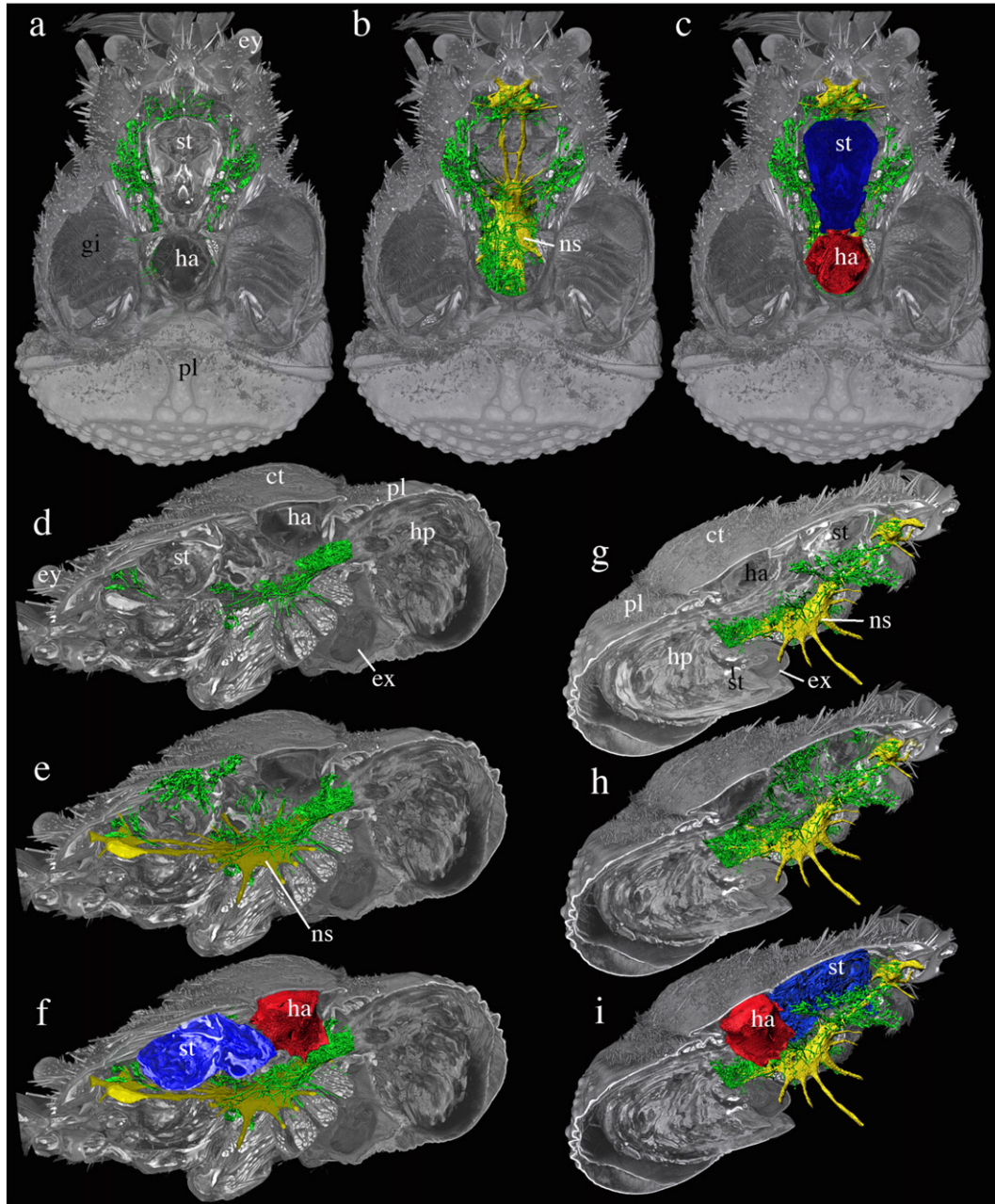


Fig. 4. Reconstruction of the rhizocephalan interna of *Briarosaccus tenellus* in the cephalothorax region in the small lithodid crab *Hapalogaster mertensii* using MicroCT; the host being virtually cut in different angles. The interna in the cephalothorax is marked in green, the interna in the pleon has not been highlighted. (a–c) Dorsal side view of the host; (d–f) left side view; (g–i) right side view; note the parasites externa on the ventral side of the pleon, which is cut in the region of the stalk that connects to the interna. (a, d, g) Interna visible between the host's organs, with part of the crab's body cut. (b, e, h) Stomach of the host virtually removed to give access to the underlying interna. Nervous system marked in yellow. (c, f, i) nervous system (yellow), heart (red), and stomach (blue) highlighted. Abbreviations: ct, cephalothorax; ey, eye; ex, externa; gi, gills; ha, heart; hp, hepatopancreas; ns, nervous system; pl, pleon; st, stalk.

2001; Pérez, 1937), has numerous side branches which themselves are simple or only moderately branched (Fig. 3), giving the interna a large surface area to absorb nutrition. The roots are highly interwoven into the crab's organs, in particular the tubular hepatopancreas (Fig. 2), which takes up the main volume of the hermit crab's pleon. The roots do not penetrate into the muscles of the hermit crab host, and are neither found in the ventral pleonal muscles, nor in the thoracic appendages. Beside *P. boschmai*, where the interna was hardly discriminable in the two scanned specimen, a typical root system as previously described for *P. paguri* could be confirmed for all other investigated *Peltogaster* species.

3.3. The interna of *B. tenellus*

As in *Peltogaster* spp., also in *B. tenellus* the main volume of the interna is present in the pleon of the host, highly interwoven into the tubules of the hepatopancreas. The roots of *B. tenellus* appear to be more evenly distributed in the pleon of its host, *H. mertensii*, than it is the case in *Peltogaster*, and no distinct structure into a "horses' tail" as in the pleonal region of *Peltogaster* is visible. The roots inside the cephalothorax are clearly visible in the most successful of the scanned *H. mertensii* specimens, which is shown in Fig. 4. The rootlets are ramifying intensively between the organs in the cephalothorax (Fig. 4 c, f,

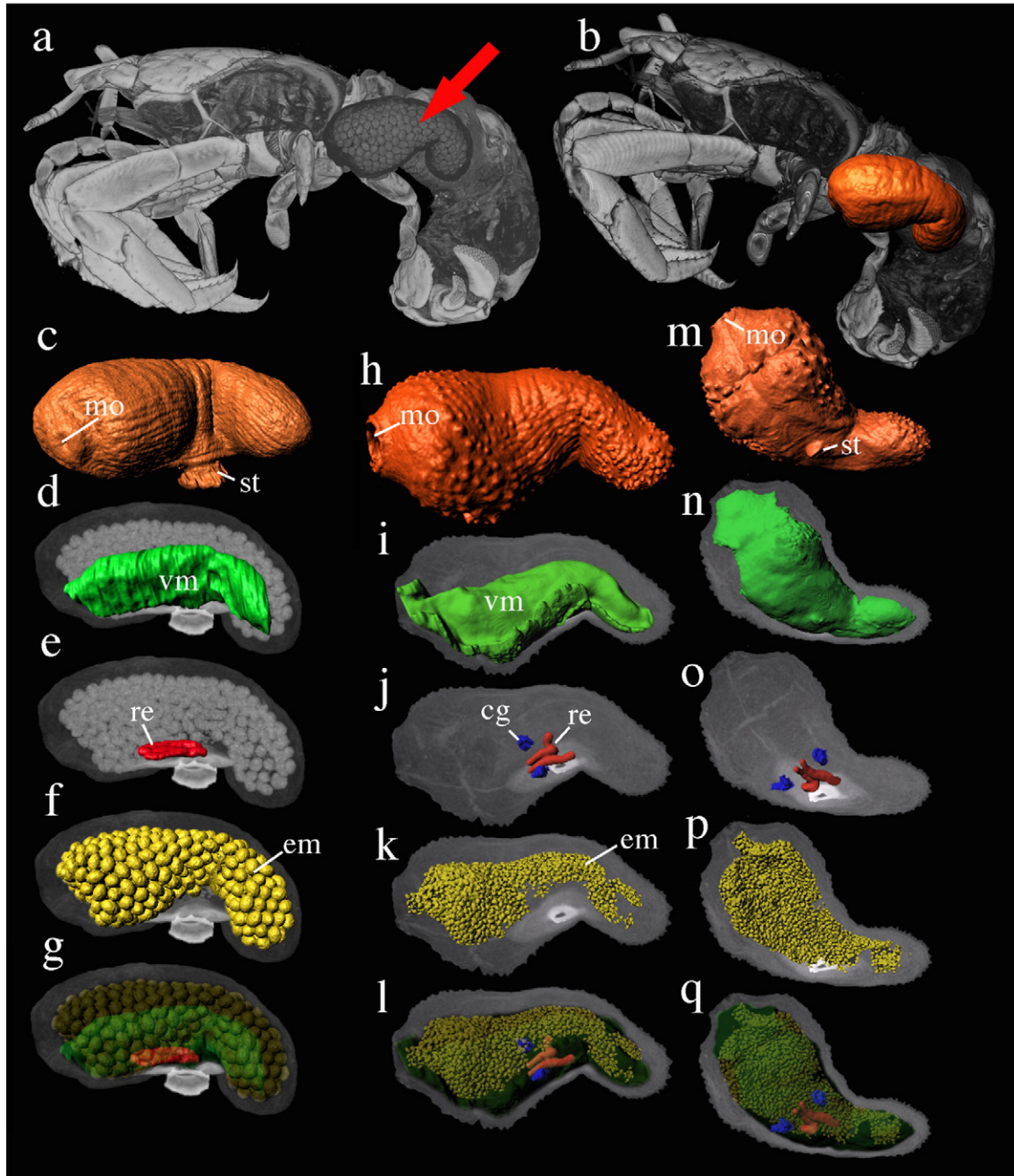


Fig. 5. The externae of the two rhizocephalan species *Peltogaster boschmai* (a–g) and *Peltogaster curvatus* (h–q) reconstructed using MicroCT. (a) Lateral view of the host, the hermit crab *Discorsopagurus schmitti*, with the externa in situ. The arrow indicates the externa. Note the visibility of eggs in the mantle cavity though the mantle, which appears transparent through MicroCT. (b) The externa of *P. boschmai* in situ on its host, with the mantle surface marked in orange. (c–g) Lateral view of the externa of *P. boschmai*, with the different organs highlighted: (c) external mantle surface (d) visceral mass; (e) receptacles; (f) egg mass; (g) visceral mass, receptacles, and egg mass combined. (h–q) Externa of *P. curvatus*, with the different organs highlighted: (h–l) ventral view; (m–q) dorsal view; (h, m) external mantle surface; note the spines which are characteristic for this species; (l, n) visceral mass; (j, o) receptacles and colleteric glands; (k, p) egg mass; (l, q) visceral mass, receptacles, colleteric glands, and egg mass combined. Abbreviations: cg, colleteric glands; em, egg mass; mo, mantle opening; re, receptacles; st, stalk; vm, visceral mass.

i) and appear to be especially affiliated to the central nervous system (Fig. 4b, e, h), as it has been documented for the genus *Briarosaccus* before (Sparks and Morado, 1986). The interna in the cephalothorax consists of long filamentous roots, which are spreading through the cephalothorax and are associated with the nervous system; and more compact branching, mycelia like roots, which are mainly found in the more vacant lateral regions under the carapace. As in *Peltogaster*, the interna of *B. tenellus* appears not to invade the musculature of its host.

H. mertensii is a small lithodid crab (Fig. 1a, b) and closely related to the large king crabs. The taxon evolved from a pagurid hermit crab ancestor (Bracken-Grissom et al., 2013; Cunningham et al., 1992; Keiler et al., 2013; Keiler et al., 2015b; Reimann et al., 2011), and as their hosts, also the rhizocephalan parasite taxa *Peltogaster* and *Briarosaccus* appear to be closely related (Boschma, 1930). While the genus *Peltogaster* parasitizes pagurid hermit crabs, the genus *Briarosaccus* is parasitizing lithodid crabs. The characteristic horses' tail structure of the *Peltogaster* interna, present in the pleon of the hermit crab hosts, is not found in *Briarosaccus* spp. (Noever et al., in press; Shirley et al., 1985; Sparks and Morado, 1986), but also here the main volume part of the interna is located in the pleon. The absence of the clear split of the interna of *Briarosaccus* into an anterior and posterior trunk inside the pleon might be related to the morphological change of its hosts due to the carcinization in the evolution of the Lithodidae. Instead of possessing an elongated pleon, which is protected by a gastropod shell as in most hermit crabs, the Lithodidae have a reduced pleon, which is inflexed under the cephalothorax in a crab-like manner (Lemaitre et al., 2007; Richter and Scholz, 1994; Scholtz, 2014). As in the Paguridae, the pleon of king crabs houses the main portion of the hepatopancreas (Anker and Paulay, 2013; Keiler et al., 2013; Keiler et al., 2015b). The hepatopancreas appears to be the target for the major part of the interna, both in *Peltogaster* and *Briarosaccus*. The structural organization of the interna between these two genera is thus directly comparable and structural differences might merely reflect the hosts' anatomy due to the carcinization of the Lithodidae.

3.4. Reconstruction of the rhizocephalan externa using MicroCT

In addition to the reconstructions of the rhizocephalan interna, which was the main target of this study, we also present 3D reconstructions of the parasite's reproductive body, the externa (Fig. 5). Morphological structures, like the extension of the mantle cavity with the containing egg mass (Fig. 5f, k, p), or the visceral mass (Fig. 5d, i, n) with the containing receptacles (Fig. 5e, j, o), are easily visible. The exact position of the receptacles can easily be observed, and in some specimens even the colleteric glands are visible in the visceral mass (Fig. 5j, o). Reconstructions of the externa of two *Peltogaster* species, *P. boschmai* (Fig. 5a–g) and *P. curvatus* (Fig. 5h–p), are shown.

For rhizocephalan taxonomy a thorough examination of the externa is often requested. Using traditionally dissection methods this entails the damaging of the examined specimen, via gross dissection or histological sectioning. MicroCT provide an alternative, non-disruptive method for morphological/taxonomical investigations of the rhizocephalan externa. This might especially be valuable when dealing with rare museum material where only one or a few specimens are available.

4. Conclusions

The present study is the first that uses MicroCT to demonstrate the in situ organization of a rhizocephalan interna inside the integument of an intact crustacean host. It demonstrates that MicroCT is excellently suited for describing the intricate, structural relationship between a rhizocephalan parasite, and the internal organs of its host. Further knowledge about the interna is essential for our understanding of the evolution of this extremely modified parasite taxon, and how the taxon developed from a suspension-feeding ancestor to a parasite

with an extremely aberrant life cycle and morphology (Glenner and Høeg, 2002; Glenner et al., 2000). In particular, MicroCT will be useful when comparing the structural organization of rhizocephalan species from a wider range of species. We also demonstrate that MicroCT is a well suitable method for morphological and taxonomical examination of the parasite's externa, which usually has to be destroyed under examination.

This technique has the potential to be an extremely useful tool in understanding the intricacies of these and other host-parasite systems. It should also be a suitable method to estimate the relative biomass of parasite versus host tissue, due to the almost artificial three-dimensional projection of both, host and parasite.

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